Methods for Sterilizing Preparations of Digestive Enzymes

Field of the Invention

The present invention relates to methods for sterilizing preparations of digestive enzymes to reduce the level of one or more active biological contaminants or pathogens therein, such as viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites. The present invention particularly relates to methods of sterilizing preparations of digestive enzymes, such as trypsin, α-galactosidase and iduronate 2-sulfatase, with irradiation.

Background of the Invention

The principal foods upon which an organism, such as a human, survives can be broadly categorized as carbohydrates, fats and proteins. These substances, however, are useless as nutrients without the process of digestion to break down foods.

Digestion of carbohydrates begins in the mouth and stomach. Saliva contains the enzyme ptyalin (an alpha-amylase), which hydrolyses starch into maltose and other small polymers of glucose. The pancreatic alpha-amylase is similar to the salivary ptyalin, but several times as powerful. Therefore, soon after chyme empties into the duodenum and mixes with pancreatic juice, virtually all of the starches are converted into disaccharides and small glucose polymers. These disaccharides and small glucose polymers are hydrolysed into monosaccharides by intestinal epithelial enzymes.

Digestion of proteins begins in the stomach. The enzyme pepsin, which is produced in the stomach, digests collagen, a major constituent of the intercellular connective tissue of meats. This enzymatic reaction is essential so that other digestive enzymes can penetrate meats and digest the cellular proteins. Consequently, in people who lack peptic activity in the stomach, the

ingested meats are not well penetrated by these other digestive enzymes and so are poorly absorbed.

Most protein digestion results from the actions of the pancreatic proteolytic enzymes. Proteins leaving the stomach in the form of proteoses, peptones and large polypeptides are digested into dipeptides, tripeptides and the like by pancreatic proteolytic enzymes or polypeptidases. Trypsin and chymotrypsin split protein molecules into smaller polypeptides at specific peptide linkages, while carboxypolypeptidase cleaves amino acids from the carboxyl ends of polypeptides. Proelastase gives rise to elastase, which in turn digests the elastin fibers that hold together most meat.

Further digestion of polypeptides takes place in the intestinal lumen. Aminopolypeptidase and several polypeptidases split large polypeptides into dipeptides, tripeptides and amino acids, which are transported into the enterocytes that line the intestinal villi. Inside the enterocytes, other polypeptidases split the remaining peptides into their constituent amino acids, which then enter the blood.

Digestion of fats first requires emulsification by bile acids and lecithin, which increase the surface area of the fats up to 1000-fold. Because lipases are water-soluble digestive enzymes that can bind only on the surface of a fat globule, this emulsification process is important for the complete digestion of fat. The most important digestive enzyme in the digestion of triglycerides is pancreatic lipase, which breaks these down into free fatty acids and 2-monoglycerides. After these free fatty acids and monoglycerides enter the enterocytes, they are generally recombined into new triglyerides. A few monoglycerides, however, are further digested by intracellular lipases into free fatty acids.

Digestion therefore continues after the breakdown and uptake of nutrients into the various cells of the body. Intracellular enzymes, such as intracellular lipases, are involved in the uptake, breakdown, transport, storage, release, metabolism and catabolism of nutrients into forms required and useable by the cell(s) of an organism at various places and times. This includes storage of lipids and their metabolism into energy sources as well as their catabolism

and synthesis into other useful compounds. Digestion may also occur as a part of an organism's normal process(es) of tissue generation and regeneration or repair of degraded, damaged or abnormal tissue(s) or molecules. It may also be a feature of or result from apoptosis, immune reactions, infections, neoplasms and other abnormal or disease states of an organism.

Preparations of digestive enzymes are therefore often provided therapeutically to humans and animals.

For example, in cases of pancreatitis and lack of pancreatic secretion, preparations of certain pancreatic enzymes, including combinations of lipase, protease and amylase (such as Creon™, Cotazym™, Donnazyme™, Ku-Zyme™ HP, Pancrease™ and Pancrease™ MT, Ultrase™ and Ultrase™ MT, Viokase™, and Zymase™) and combinations of lipase, protease, amylase and cellulase (such as Ku-Zyme™ and Kutrase™), are administered to ensure proper patient nutrition. The digestive enzymes of particular interest, for example in replacement therapy in humans and animals, therefore include pancreatic digestive enzymes, such as trypsin and chymotrypsin, and functional mutants, variants and derivatives thereof.

Trypsin is an enzyme that acts to degrade protein; it is often referred to as a digestive enzyme, or proteinase. In the digestive process, trypsin acts with the other proteinases to break down dietary protein molecules to their component peptides and amino acids. Trypsin continues the process of digestion (begun in the stomach) in the small intestine where a slightly alkaline environment (about pH 8) promotes its maximal enzymatic activity. Trypsin, produced in an inactive form by the pancreas, is remarkably similar in chemical composition and in structure to the other chief pancreatic proteinase, chymotrypsin. Both enzymes also appear to have similar mechanisms of action; residues of histidine and serine are found in the active sites of both. The chief difference between the two molecules seems to be in their specificity, that is, each is active only against the peptide bonds in protein molecules that have carboxyl groups donated by certain amino acids. For trypsin these amino acids are arginine and lysine, for chymotrypsin they are tyrosine, phenylalanine, tryptophan, methionine, and leucine. Trypsin is the most discriminating of all the digestive enzymes in terms of the restricted number of chemical bonds that it will attack.

Preparations of other digestive enzymes, such as glycosidases, are likewise administered therapeutically to human patients. For example, Fabry disease is an X-linked recessive glycolipid storage disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A. Clinical manifestations of Fabry disease included recurrent episodes severe pain and progressive renal, cardiac and cerebrovascular deterioration with death usually occurring in the fourth to sixth decade of life. Enzyme replacement therapy by infusion of a preparation of α-galactosidase A has been tested and found to be a promising potential therapy for this condition (Schiffmann, *et al.*, "Enzyme Replacement Therapy in Fabry Disease: A Randomized Controlled Trial." *JAMA*, June 6, 2001, Vol. 285, No. 21, pp. 2743-2749.).

Glycogen Storage Disease Type II (also known as Acid Maltase Deficiency or Pompe Disease) is another genetically transmitted storage disorder. In GSD-II, the patient suffers from a deficiency of acid maltase enzyme, which breaks down glycogen in muscle cells. Clinical manifestations of GSD-II include progressive muscle weakness due to a build up of glycogen in muscle tissues, eventually resulting in respiratory and/or cardiac failure. Preparations of glycosidases, or functional mutants or variants or derivatives thereof, are therefore also of particular interest for therapeutic use.

Niemann-Pick Disease is also a genetically transmitted metabolic disorder in which harmful quantities of a fatty substance, sphingomyelin, accumulate in the spleen, liver, lungs, bone marrow and brain. Patients suffer from a deficiency of sphingomyelinases, which initiates the biodegradation of sphinogmyelin. Clinical manifestations include enlargement of the spleen and liver, and frequently results in death, particularly for pediatric patients.

Gaucher's Disease is a somewhat-similar genetically transmitted disorder, in which harmful quantities of another fatty substance, glucocerebroside, accumulate in the spleen, liver, lungs, bone marrow and brain. Patients suffer from a deficiency in β-glucocerebrosidase, which catalyzes the first step in the biodegradation of glucocerebroside, which arises from the biodegradation of old red and white blood cells. Clinical manifestations include enlargement of the spleen and liver, low blood platelets, fatigue and, in certain forms, progressive brain damage. Enzyme replacement therapy by infusion of a preparation of a modified form of glucocerebrosidase, known as algucerase (Ceredase™) has been tested and found to be a promising potential therapy for this condition (Barton, *et al.*, "Replacement Therapy for Enzyme

Deficiency: Macrophage-targeted Glucocerebrosidase for Gaucher's Disease." *New Engl. J. Med.*, May 23, 1991.).

Mucopolysaccharidoses are a group of inherited metabolic disorders caused by a deficiency in the lysosomal enzymes needed to break down mucopolysaccharides, long chains of sugar molecules used to build connective tissue and organs in the body. A deficiency in one or more of these enzymes cases a build up of excess amount in the body, causing progressive damage and eventual death. Among these disorders are Hurler, Scheie and Hurler/Scheie syndromes (the most severe form, occurs in infancy with death resulting before age 10 years, symptoms include clouding of the cornea and progressive physical and mental disability, caused by a deficiency in α-L-iduronidase), Hunter syndrome (affects juveniles with death usually resulting by age 15 years, symptoms include joint stiffness, mental deterioration, dwarfing and progressive deafness, caused by a deficiency in iduronate-2-sulfatase), Sanfillipo syndrome (death usually occurs by late teens, symptoms include progressive dementia and mental deterioration in childhood, caused by a deficiency in heparan N-sulfatase, α-N-acetylglucosaminadase, acetyl-CoA-glucosaminide acetyltransferase and/or N-acetylglucosamine-6-sulfatase), Morquio syndrome (appears in infancy, symptoms include severe dwarfing and corneal clouding, cardiac or respiratory disease may cause death in third or fourth decade of like, caused by a deficiency in galactosamine-6-sulfatase and/or β-galactosidase), Maroteauz-Lamy syndrome (resembles Hurler syndrome, onset in infancy, but no mental disability, death usually occurs in second or third decade of life, caused by a deficiency in arylsulfatase B), and Sly disease (symptoms include corneal clouding, skeletal irregularities, and enlargement of the liver and spleen, caused by a deficiency in β-glucuronidase). Hunter syndrome is particularly linked to a deficiency in iduronate-2-sulfatase, which catalyzes the breakdown of heparan sulfate and dermatan sulfate, and it has been suggested that this condition can be treated by administration of variant forms of the enzyme (U.S. Patent No. 6,153,188). The digestive of particular interest, for example in therapy in humans and animals, therefore also include iduronate-2-sulfatase and functional mutants, variants and derivatives thereof.

Multiple Sulfatase Deficiency (also known as Disorder of Confication 13 or Mucosulfatidosis) is another hereditary metabolic disorder characterized by impairment of all known sulfatase enzymes (including arylsulfatases A, B and C, two steroid sulfatases and four

other sulfatases). Clinical manifestations include coarse facial features, deafness, an enlarged liver and spleen, abnormalities of the skeleton (including lumbar kyphosis) and dry, scaly skin (ichthyosis).

Similarly, preparations of digestive enzymes are administered to humans and animals to improve nutrition.

For example, in cases of lactose intolerance, preparations of lactase (such as Lactaid™) are administered to humans in need thereof. Lactose intolerance is characterized by gastrointestinal discomfort, including gas, bloating, crampls and diarrhea, after the consumption of milk or milk-containing products. The digestive enzymes of particular interest, for example in therapy in humans and animals, therefore also include lactase and functional mutants, variants and derivatives thereof.

Likewise, preparations of galactosidases (such as Beano™ or Nutritek™ Alpha Galactosidase) are administered to humans in need thereof. Such products improve digestion of sugars found in foods including legumes and cruciferous vegetables and reduce effects generally associated with the foods, such as gas and bloating.

Preparations of digestive enzymes that are prepared for human, veterinary, diagnostic and/or experimental use may contain unwanted and potentially dangerous biological contaminants or pathogens, such as viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites. Consequently, it is of utmost importance that any biological contaminant in the preparation be inactivated before the product is used. This is especially critical when the preparation is to be administered directly to a patient, for example in human therapy corrected or treated by intravenous, intramuscular or other forms of injection. This is also critical for the various preparations that are prepared in media or via culture of cells or recombinant cells which contain various types of plasma and/or plasma derivatives or other biological materials or are used to prepare biological materials for human use and which may be subject to mycoplasma, prion, bacterial, viral and/or other biological contaminants or pathogens.

Most procedures for producing preparations of digestive enzymes have involved methods that screen or test the preparation for one or more particular biological contaminants or pathogens rather than removal or inactivation of the contaminant(s) and/or pathogen(s) from the preparation. Preparations that test positive for a biological contaminant or pathogen are merely not used. Examples of screening procedures include the testing for a particular virus in human blood from blood donors. Such procedures, however, are not always reliable and are not able to detect the presence of certain viruses, particularly in very low numbers, and in the case of as yet unknown viruses or other contaminants or pathogens that may be in blood. This reduces the value or certainty of the test in view of the consequences associated with a false negative result. False negative results can be life threatening in certain cases, for example in the case of Acquired Immune Deficiency Syndrome (AIDS). Furthermore, in some instances it can take weeks, if not months, to determine whether or not the preparation is contaminated. Therefore, it would be desirable to apply techniques that would kill or inactivate biological contaminants and pathogens during and/or after manufacturing the preparation of digestive enzymes.

In conducting experiments to determine the ability of technologies to inactivate viruses, the actual viruses of concern are seldom utilized. This is a result of safety concerns for the workers conducting the tests, and the difficulty and expense associated with the containment facilities and waste disposal. In their place, model viruses of the same family and class are used.

In general, it is acknowledged that the most difficult viruses to inactivate are those with an outer shell made up of proteins, and that among these, the most difficult to inactivate are those of the smallest size. This has been shown to be true for gamma irradiation and most other forms of radiation as these viruses' diminutive size is associated with a small genome. The magnitude of direct effects of radiation upon a molecule are directly proportional to the size of the molecule, that is the larger the target molecule, the greater the effect. As a corollary, it has been shown for gamma-irradiation that the smaller the viral genome, the higher the radiation dose required to inactive it.

Among the viruses of concern for both human and animal-derived preparations, the smallest, and thus most difficult to inactivate, belong to the family of Parvoviruses and the slightly larger protein-coated Hepatitis virus. In humans, the Parvovirus B19, and Hepatitis A

are the agents of concern. In porcine-derived materials, the smallest corresponding virus is Porcine Parvovirus. Since this virus is harmless to humans, it is frequently chosen as a model virus for the human B19 Parvovirus. The demonstration of inactivation of this model parvovirus is considered adequate proof that the method employed will kill human B19 virus and Hepatitis A, and by extension, that it will also kill the larger and less hardy viruses such as HIV, CMV, Hepatitis B and C and others.

More recent efforts have focussed on methods to remove or inactivate contaminants in the products. Such methods include heat treating, filtration and the addition of chemical inactivants or sensitizers to the product.

Heat treatment requires that the product be heated to approximately 60°C for about 70 hours which can be damaging to sensitive products. In some instances, heat inactivation can actually destroy 50% or more of the biological activity of the product.

Filtration involves filtering the product in order to physically remove contaminants. Unfortunately, this method may also remove products that have a high molecular weight. Further, in certain cases, small viruses and similarly sized contaminants and pathogens, such as prions, may not be removed by the filter.

The procedure of chemical sensitization involves the addition of noxious agents which bind to the DNA/RNA of the virus and which are activated either by UV or other radiation. This radiation produces reactive intermediates and/or free radicals which bind to the DNA/RNA of the virus, break the chemical bonds in the backbone of the DNA/RNA, and/or cross-link or complex it in such a way that the virus can no longer replicate. This procedure requires that unbound sensitizer is washed from products since the sensitizers are toxic, if not mutagenic or carcinogenic, and cannot be administered to a patient.

Irradiating a product with gamma radiation is another method of sterilizing a product. Gamma radiation is effective in destroying viruses and bacteria when given in high total doses (Keathly *et al.*, "Is There Life After Irradiation? Part 2," *BioPharm* July-August, 1993, and Leitman, USe of Blood Cell Irradiation in the Prevention of Post Transfusion Graft-vs-Host

Disease," *Transfusion Science 10*:219-239 (1989)). The published literature in this area, however, teaches that gamma radiation can be damaging to radiation sensitive products, such as blood, blood products, enzymes, protein and protein-containing products. In particular, it has been shown that high radiation doses are injurious to red cells, platelets and granulocytes (Leitman). U.S. Patent No. 4,620,908 discloses that protein products must be frozen prior to irradiation in order to maintain the viability of the protein product. This patent concludes that "[i]f the gamma irradiation were applied while the protein material was at, for example, ambient temperature, the material would be also completely destroyed, that is the activity of the material would be rendered so low as to be virtually ineffective". Unfortunately, many sensitive biological materials, such as monoclonal antibodies (Mab), may lose viability and activity if subjected to freezing for irradiation purposes and then thawing prior to administration to a patient.

In view of the difficulties discussed above, there remains a need for methods of sterilizing preparations of one or more digestive enzymes that are effective for reducing the level of active biological contaminants or pathogens without an adverse effect on the preparation.

Summary of the Invention

Accordingly, it is an object of the present invention to provide methods of sterilizing preparations of digestive enzymes by reducing the level of active biological contaminants or pathogens without adversely effecting the preparation. Other objects, features and advantages of the present invention will be set forth in the detailed description of preferred embodiments that follows, and in part will be apparent from the description or may be learned by practice of the invention. These objects and advantages of the invention will be realized and attained by the compositions and methods particularly pointed out in the written description and claims hereof.

In accordance with these and other objects, a first embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising irradiating the preparation of one or more digestive enzymes

with radiation for a time effective to sterilize the material at a rate effective to sterilize the material and to protect the material from radiation.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) adding to a preparation of one or more digestive enzymes at least one stabilizer in an amount effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the material.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the residual solvent content of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the temperature of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes a stabilizing process selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an

effective rate for a time effective to sterilize the preparation of one or more digestive enzymes, wherein the stabilizing process and the rate of irradiation are together effective to protect the preparation of one or more digestive enzymes from radiation.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes at least two stabilizing processes selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes, wherein the stabilizing processes may be performed in any order and are together effective to protect the preparation of one or more digestive enzymes from radiation.

The invention also provides a biological composition comprising at least one preparation of one or more digestive enzymes and a least one stabilizer in an amount effective to preserve the preparation of one or more digestive enzymes for its intended use following sterilization with radiation.

The invention also provides a biological composition comprising at least one preparation of one or more digestive enzymes in which the residual solvent content has been reduced to a level effective to preserve the preparation of one or more digestive enzymes for its intended use following sterilization with radiation.

The invention also provides a biological composition comprising at least one preparation of one or more digestive enzymes and at least one stabilizer in which the residual solvent content has been reduced and wherein the amount of stabilizer and level of residual solvent content are together effective to preserve the preparation of one or more digestive enzymes for its intended use following sterilization with radiation.

The invention also provides a biological composition comprising at least one preparation of one or more digestive enzymes wherein the total protein concentration of the preparation is effective to preserve the preparation of one or more digestive enzymes for its intended use following sterilization with radiation.

Brief Description of the Drawings

Figures 1A-1B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer and at varying levels of residual solvent content.

Figure 2 is a graph showing the activity of liquid or lyophilized trypsin following gamma irradiation in the presence of a stabilizer and at varying pH levels.

Figures 3A-3B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer.

Figures 4A-4B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer and at varying levels of residual solvent content.

Figures 5A-5B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer and at varying levels of residual solvent content.

Figure 6 is a graph showing the activity of trypsin suspended in polypropylene glycol following gamma irradiation at varying levels of residual solvent content.

Figure 7 is a graph showing the activity of trypsin following gamma irradiation in an aqueous solution at varying concentrations of stabilizers.

Figures 8A-8B are gels showing the protective effect of ascorbate (200 mM) and a combination of ascorbate (200 mM) and Gly-Gly (200 mM) on two different frozen enzyme preparations (a glycosidase and a sulfatase).

Figure 9 is a graph showing the protective effect of stabilizers on a frozen glycosidase preparation.

Figure 10 shows the protective effect of ascorbate on two different lyophilized enzyme preparations (a glycosidase and a sulfatase).

Figures 11A-11C are gels showing the protective effect of ascorbate (200 mM) and a combination of ascorbate (200 mM) and Gly-Gly (200 mM) on a lyophilized glycosidase preparation.

Detailed Description of the Preferred Embodiments

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein are intended to have the same meaning as is commonly understood by one of ordinary skill in the relevant art.

As used herein, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used herein, the term "preparation of one or more digestive enzymes" is intended to mean any preparation derived or obtained from a living organism that contains one or more of enzymes involved in the breakdown or conversion of one substance into a second substance, particularly protein(s), lipid(s) and/or cabohydrate(s). Illustrative examples of digestive enzymes include, but are not limited to, intracellular and intercellular enzymes produced by, present in or introduced into the digestive tract of any living organism, or involved in the metabolism, catabolism, storage and mobilization of externally or internally-derived nutrients or the breakdown products of tissue and/or cellular repair, regeneration, or removal, such as the

following: pancreatic enzymes, including pancreatic proteolytic enzymes, such as trypsin and chymotrypsin, pancreatic lipase and pancreatic amylase; salivary enzymes, such as ptyalin; intestinal enzymes, including intestinal polypeptidases, intestinal amylases and intestinal lipases; glycosidases, such as α-galactosidase; and sulfatases, such as iduronodate-2-sulfatase.

As used herein, the term "sterilize" is intended to mean a reduction in the level of at least one active biological contaminant or pathogen found in the preparation being treated according to the present invention.

As used herein, the term "biological contaminant or pathogen" is intended to mean a contaminant or pathogen that, upon direct or indirect contact with a preparation of one or more digestive enzymes, may have a deleterious effect on the digestive enzymes or upon a recipient thereof. Such biological contaminants or pathogens include the various viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites known to those of skill in the art to generally be found in or infect preparations of digestive enzymes. Examples of biological contaminants or pathogens include, but are not limited to, the following: viruses, such as human immunodeficiency viruses and other retroviruses, herpes viruses, filoviruses, circoviruses, paramyxoviruses, cytomegaloviruses, hepatitis viruses (including hepatitis A, B and C), pox viruses, toga viruses, Epstein-Barr viruses and parvoviruses; bacteria, such as *Escherichia*, *Bacillus*, *Campylobacter*, *Streptococcus* and *Staphalococcus*; nanobacteria; parasites, such as *Trypanosoma* and malarial parasites, including *Plasmodium* species; yeasts; molds; mycoplasmas and ureaplasmas; chlamydia; rickettsias, such as *Coxiella burnetti*; and prions and similar agents responsible, alone or in combination, for one or more of the disease states known as transmissible spongiform encephalopathies (TSEs) in mammals, such as scrapie, transmissible mink encephalopathy, chronic wasting disease (generally observed in mule deer and elk), feline spongiform encephalopathy, bovine spongiform encephalopathy (mad cow disease); Creutzfeld-Jakob disease (including variant or new variant CJD), Fatal Familial Insomnia; Gerstmann-Straeussler-Scheinker syndrome; kuru; and Alpers syndrome. As used herein, the term "active biological contaminant or pathogen" is intended to mean a biological contaminant or pathogen

that is capable of causing a deleterious effect, either alone or in combination with another factor, such as a second biological contaminant or pathogen or a native protein (wild-type or mutant) or antibody, in the preparation of digestive enzymes and/or a recipient thereof.

As used herein, the term "a biologically compatible solution" is intended to mean a solution to which a preparation of one or more digestive enzymes may be exposed, such as by being suspended or dissolved therein, and remain viable, *i.e.*, retain its essential biological and physiological characteristics.

As used herein, the term "a biologically compatible buffered solution" is intended to mean a biologically compatible solution having a pH and osmotic properties (*e.g.*, tonicity, osmolality and/or oncotic pressure) suitable for maintaining the integrity of the material(s) therein. Suitable biologically compatible buffered solutions typically have a pH between 4 and 8.5 and are isotonic or only moderately hypotonic or hypertonic. Biologically compatible buffered solutions are known and readily available to those of skill in the art.

As used herein, the term "stabilizer" is intended to mean a compound or material that reduces damage to the biological material being irradiated to a level that is insufficient to preclude the safe and effective use of the material. Illustrative examples of stabilizers include, but are not limited to, the following: antioxidants; free radical scavengers, including spin traps; combination stabilizers, *i.e.* stabilizers which are effective at quenching both Type I and Type II photodynamic reactions; and ligands, such as heparin, that stabilize the molecules to which they bind. Preferred examples of stabilizers include, but are not limited to, the following: ethanol; acetone; fatty acids, including 6,8-dimercapto-octanoic acid (lipoic acid) and its derivatives and analogues (alpha, beta, dihydro, bisno and tetranor lipoic acid), thioctic acid, 6,8-dimercapto-octanoic acid, dihydrolopoate (DL-6,8-dithioloctanoic acid methyl ester), lipoamide, bisonor methyl ester and tatranor-dihydrolipoic acid, furan fatty acids, oleic and linoleic and palmitic acids and their salts and derivatives; flavonoids, phenylpropaniods, and flavenols, such as quercetin, rutin and its derivatives, apigenin, aminoflavone, catechin, hesperidin and, naringin; carotenes, including beta-carotene; Co-Q10; xanthophylls; polyhydric alcohols, such as glycerol, mannitol; sugars, such as xylose, glucose, ribose, mannose, fructose and trehalose; amino acids

and derivatives thereof, such as histidine, N-acetylcysteine (NAC), glutamic acid, tryptophan, sodium caprylate, N-acetyl tryptophan and methionine; azides, such as sodium azide; enzymes, such as Superoxide Dismutase (SOD) and Catalase; uric acid and its derivatives, such as 1,3-dimethyluric acid and dimethylthiourea; allopurinol; thiols, such as glutathione and reduced glutathione and cysteine; trace elements, such as selenium; vitamins, such as vitamin A, vitamin C (including its derivatives and salts such as sodium ascorbate and palmitoyl ascorbic acid) and vitamin E (and its derivatives and salts such as tocopherol acetate and alpha-tocotrienol); chromanol-alpha-C6; 6-hydroxy-2,5,7,8-tetramethylchroma-2 carboxylic acid (Trolox) and derivatives; extraneous proteins, such as gelatin and albumin; tris-3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186); citiolone; puercetin; chrysin; dimethyl sulfoxide (DMSO); piperazine diethanesulfonic acid (PIPES); imidazole; methoxypsoralen (MOPS); 1,2-dithiane-4,5-diol; reducing substances, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT); cholesterol; probucol; indole derivatives; thimerosal; lazaroid and tirilazad mesylate; proanthenols; proanthocyanidins; ammonium sulfate; Pegorgotein (PEG-SOD); N-*tert*-butyl-alpha-phenylnitrone (PBN); 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol); mixtures of ascorbate, urate and Trolox C (Asc/urate/Trolox C); proteins and peptides, such as glycylglycine and carnosine, in which each amino acid may be in its D or L form; diosmin; pupurogalin; gallic acid and its derivatives including but not limited to propyl gallate, sodium formaldehyde sulfoxylate and silymarin. Particularly preferred examples include single stabilizers or combinations of stabilizers that are effective at quenching both Type I and Type II photodynamic reactions and volatile stabilizers, which can be applied as a gas and/or easily removed by evaporation, low pressure and similar methods.

As used herein, the term "residual solvent content" is intended to mean the amount or proportion of freely available liquid in the preparation of one or more digestive enzymes. Freely available liquid means the liquid, such as water or an organic solvent (*e.g.* ethanol, isopropanol, acetone, polyethylene glycol, etc.), present in the preparation being sterilized that is not bound to or complexed with one or more of the non-liquid components of the preparation. Freely available liquid includes intracellular water. The residual solvent contents related as water referenced herein refer to levels determined by the FDA approved, modified Karl Fischer

method (Meyer and Boyd, *Analytical Chem.*, *31*:215-219, 1959; May, *et al.*, *J. Biol. Standardization, 10*:249-259, 1982; Centers for Biologics Evaluation and Research, FDA, Docket No. 89D-0140, 83-93; 1990) and/or by near infrared spectroscopy. Quantitation of the residual levels of other solvents may be determined by means well known in the art, depending upon which solvent is employed. The proportion of residual solvent to solute may also be considered to be a reflection of the concentration of the solute within the solvent. When so expressed, the greater the concentration of the solute, the lower the amount of residual solvent.

As used herein, the term "sensitizer" is intended to mean a substance that selectively targets viral, bacterial, nanobacterial, mold, yeast, fungal, prion and/or parasitic contaminants or pathogens, rendering them more sensitive to inactivation by radiation, therefore permitting the use of a lower rate or dose of radiation and/or a shorter time of irradiation than in the absence of the sensitizer. Illustrative examples of suitable sensitizers include, but are not limited to, the following: psoralen and its derivatives and analogs (including 3-carboethoxy psoralens); inactines and their derivatives and analogs; angelicins, khellins and coumarins which contain a halogen substituent and a water solubilization moiety, such as quaternary ammonium ion or phosphonium ion; nucleic acid binding compounds; brominated hematoporphyrin; phthalocyanines; purpurins; porphorins; halogenated or metal atom-substituted derivatives of dihematoporphyrin esters, hematoporphyrin derivatives, benzoporphyrin derivatives, hydrodibenzoporphyrin dimaleimade, hydrodibenzoporphyrin, dicyano disulfone, tetracarbethoxy hydrodibenzoporphyrin, and tetracarbethoxy hydrodibenzoporphyrin dipropionamide; doxorubicin and daunomycin, which may be modified with halogens or metal atoms; netropsin; BD peptide, S2 peptide; S-303 (ALE compound); dyes, such as hypericin, methylene blue, eosin, fluoresceins (and their derivatives), flavins, merocyanine 540; photoactive compounds, such as bergapten; and SE peptide.

As used herein, the term "radiation" is intended to mean radiation of sufficient energy to sterilize at least some component of the irradiated preparation of one or more digestive enzymes. Types of radiation include, but are not limited to, the following: (i) corpuscular (streams of subatomic particles such as neutrons, electrons, and/or protons); and (ii) electromagnetic (originating in a varying electromagnetic field, such as radio waves, visible (both mono and

polychromatic) and invisible light, infrared, ultraviolet radiation, x-radiation, and gamma rays and mixtures thereof). Such radiation is often described as either ionizing (capable of producing ions in irradiated materials) radiation, such as gamma rays, and non-ionizing radiation, such as visible light. The sources of such radiation may vary and, in general, the selection of a specific source of radiation is not critical provided that sufficient radiation is given in an appropriate time and at an appropriate rate to effect sterilization. In practice, gamma radiation is usually produced by isotopes of Cobalt or Cesium, while X-rays are produced by machines that emit X-radiation, and electrons are often used to sterilize materials in a method known as "E-beam" irradiation that involves their production via a machine.

As used herein, the term "to protect" is intended to mean to reduce any damage to the preparation of one or more digestive enzymes being irradiated, that would otherwise result from the irradiation of that material, to a level that is insufficient to preclude the safe and effective use of the material following irradiation. In other words, a substance or process "protects" a preparation of one or more digestive enzymes from radiation if the presence of that substance or carrying out that process results in less damage to the material from irradiation than in the absence of that substance or process. Thus, a preparation of one or more digestive enzymes may be used safely and effectively after irradiation in the presence of a substance or following performance of a process that "protects" the material, but could not be used safely and effectively after irradiation under identical conditions but in the absence of that substance or the performance of that process.

B. Particularly Preferred Embodiments

A first preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising irradiating the preparation of one or more digestive enzymes with radiation for a time effective to sterilize the material at a rate effective to sterilize the material and to protect the material from radiation.

Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) adding to a preparation of one or more digestive enzymes at least one stabilizer in an amount effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the material.

Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the residual solvent content of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the temperature of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes a stabilizing process selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one

or more digestive enzymes, wherein the stabilizing process and the rate of irradiation are together effective to protect the preparation of one or more digestive enzymes from radiation.

Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes at least two stabilizing processes selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes, wherein the stabilizing processes may be performed in any order and are together effective to protect the preparation of one or more digestive enzymes from radiation.

According to certain methods of the present invention, a stabilizer is added to the preparation of one or more digestive enzymes prior to irradiation of the preparation of one or more digestive enzymes with radiation. This stabilizer is added in an amount that is effective to protect the preparation of one or more digestive enzymes from the radiation. Suitable amounts of stabilizer may vary depending upon certain features of the particular method(s) of the present invention being employed, such as the nature and characteristics of the particular preparation of one or more digestive enzymes and/or stabilizer being used, and/or the intended use of the preparation of one or more digestive enzymes being irradiated, and can be determined empirically by one skilled in the art.

According to certain methods of the present invention, the residual solvent content of the preparation of one or more digestive enzymes is reduced prior to irradiation of the preparation of one or more digestive enzymes with radiation. The residual solvent content is reduced to a level that is effective to protect the preparation of one or more digestive enzymes from the radiation. Suitable levels of residual solvent content may vary depending upon certain features of the particular method(s) of the present invention being employed, such as the nature and

characteristics of the particular preparation of one or more digestive enzymes and/or stabilizer being used, and/or the intended use of the preparation of one or more digestive enzymes being irradiated, and can be determined empirically by one skilled in the art. There may be preparations for which it is desirable to maintain the residual solvent content to within a particular range, rather than a specific value, for example when the solvent, or at least one of the solvents in a mixture, is also a stabilizer, such as an alcohol (*e.g.* ethanol) or dialkyl ketone (*e.g.* acetone).

When the solvent is water, and particularly when the preparation of one or more digestive enzymes is in a solid phase, the residual solvent content is generally less than about 15%, typically less than about 10%, more typically less than about 9%, even more typically less than about 8%, usually less than about 5%, preferably less than about 3.0%, more preferably less than about 2.0%, even more preferably less than about 1.0%, still more preferably less than about 0.5%, still even more preferably less than about 0.2% and most preferably less than about 0.08%.

The solvent may preferably be a non-aqueous solvent, more preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation, and most preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation and that has little or no dissolved oxygen or other gas(es) that is (are) prone to the formation of free-radicals upon irradiation. Volatile non-aqueous solvents are particularly preferred, even more particularly preferred are non-aqueous solvents that are stabilizers, such as ethanol and acetone.

In certain embodiments of the present invention, the solvent may be a mixture of water and a non-aqueous solvent or solvents, such as ethanol and/or acetone. In such embodiments, the non-aqueous solvent(s) is preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation, and most preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation and that has little or no dissolved oxygen or other gas(es) that is (are) prone to the formation of free-radicals upon irradiation. Volatile non-aqueous solvents are particularly preferred, even more particularly preferred are non-aqueous solvents that are stabilizers, such as ethanol and acetone.

In a preferred embodiment, when the residual solvent is water, the residual solvent content of a biological material is reduced by dissolving or suspending the biological material in a non-aqueous solvent that is capable of dissolving water. Preferably, such a non-aqueous solvent is not prone to the formation of free-radicals upon irradiation and has little or no dissolved oxygen or other gas(es) that is (are) prone to the formation of free-radicals upon irradiation.

When the biological material is in a liquid phase, reducing the residual solvent content may be accomplished by any of a number of means, such as by increasing the solute concentration. In this manner, the concentration of the biological material dissolved within the solvent may be increased to generally at least about 0.5%, typically at least about 1%, usually at least about 5%, preferably at least about 10%, more preferably at least about 15%, even more preferably at least about 20%, still even more preferably at least about 25%, and most preferably at least about 50%.

In certain embodiments of the present invention, the residual solvent content of a particular biological material may be found to lie within a range, rather than at a specific point. Such a range for the preferred residual solvent content of a particular biological material may be determined empirically by one skilled in the art.

While not wishing to be bound by any theory of operability, it is believed that the reduction in residual solvent content reduces the degrees of freedom of the preparation of one or more digestive enzymes, reduces the number of targets for free radical generation and may restrict the solubility or diffusion of these free radicals. Similar results might therefore be achieved by lowering the temperature of the preparation of one or more digestive enzymes below its eutectic point or below its freezing point, or by vitrification to likewise reduce the degrees of freedom of the preparation of one or more digestive enzymes. These results may permit the use of a higher rate and/or dose of radiation than might otherwise be acceptable. Thus, the methods described herein may be carried out at any temperature that doesn't result in an unacceptable level of damage to the preparation. Preferably, the methods described herein

are performed at ambient temperature or below ambient temperature, such as below the eutectic point or freezing point of the preparation of one or more digestive enzymes being irradiated.

In accordance with the methods of the present invention, an "acceptable level" of damage may vary depending upon certain features of the particular method(s) of the present invention being employed, such as the nature and characteristics of the particular preparation of one or more digestive enzymes and/or stabilizer being used, and/or the intended use of the preparation of one or more digestive enzymes being irradiated, and can be determined empirically by one skilled in the art. An "unacceptable level" of damage would therefore be a level of damage that would preclude the safe and effective use of the preparation of one or more digestive enzymes being sterilized. The particular level of damage in a given preparation of one or more digestive enzymes may be determined using any of the methods and techniques known to one skilled in the art.

The residual solvent content of a preparation of one or more digestive enzymes may be reduced by any of the methods and techniques known to those skilled in the art for reducing solvent from a preparation of one or more digestive enzymes without producing an unacceptable level of damage to the preparation. Such methods include, but are not limited to, evaporation, concentration, centrifugal concentration, vitrification, addition of solute, lyophilization (with or without the prior addition of ascorbate) and spray-drying.

A particularly preferred method for reducing the residual solvent content of a preparation of one or more digestive enzymes is lyophilization, even more preferred is lyophilization following the addition of ascorbate.

Another particularly preferred method for reducing the residual solvent content of a preparation of one or more digestive enzymes is vitrification, which may be accomplished by any of the methods and techniques known to those skilled in the art, including the addition of solute and or additional solutes, such as sucrose, to raise the eutectic point of the biological material, followed by a gradual application of reduced pressure to the biological material in

order to remove the residual solvent, such as water. The resulting glassy material will then have a reduced residual solvent content.

According to certain methods of the present invention, the preparation of one or more digestive enzymes to be sterilized may be immobilized upon a solid surface by any means known and available to one skilled in the art. For example, the preparation of one or more digestive enzymes to be sterilized may be present as a coating or surface on a biological or non-biological substrate.

The radiation employed in the methods of the present invention may be any radiation effective for the inactivation of one or more biological contaminants or pathogens of the preparation of one or more digestive enzymes being treated. The radiation may be corpuscular, including E-beam radiation. Preferably the radiation is electromagnetic radiation, including visible light, infrared, x-radiation, UV light and mixtures of various wavelengths of electromagnetic radiation. A particularly preferred form of radiation is gamma radiation.

According to the methods of the present invention, the preparation of one or more digestive enzymes to be sterilized is irradiated with the radiation at a rate effective for the inactivation of one or more biological contaminants or pathogens of the preparation. Suitable rates of irradiation may vary depending upon certain features of the methods of the present invention being employed, such as the nature and characteristics of the particular preparation of one or more digestive enzymes being irradiated, the particular form of radiation involved and/or the particular biological contaminants or pathogens being inactivated. Suitable rates of irradiation can be determined empirically by one skilled in the art. Preferably, the rate of irradiation is constant for the duration of the sterilization procedure. When this is impractical or otherwise not desired, a variable or discontinuous irradiation may be utilized.

According to the methods of the present invention, the rate of irradiation may be optimized to produce the most advantageous combination of product recovery and time required to complete the operation. Both low ($\leq$3 kGy/hour) and high (>3 kGy/hour) rates may be utilized in the methods described herein to achieve such results.

According to a particularly preferred embodiment of the present invention, the rate of irradiation is not more than about 3.0 kGy/hour, more preferably between about 0.1 kGy/hr. and 3.0 kGy/hr, even more preferably between about 0.25 kGy/hr and 2.0 kGy/hour, still even more preferably between about 0.5 kGy/hr and 1.5 kGy/hr and most preferably between about 0.5 kGy/hr and 1.0 kGy/hr.

According to another particularly preferred embodiment of the present invention, the rate of irradiation is at least about 3.0 kGy/hr., more preferably at least about 6 kGy/hr., even more preferably at least about 16 kGy/hr., and even more preferably at least about 30 kGy/hr and most preferably at least about 45 kGy/hr or greater.

According to the methods of the present invention, the preparation of one or more digestive enzymes to be sterilized is irradiated with the radiation for a time effective for the inactivation of one or more biological contaminants or pathogens of the preparation of one or more digestive enzymes. Combined with irradiation rate, the appropriate irradiation time results in the appropriate dose of irradiation being applied to the preparation of one or more digestive enzymes. Suitable irradiation times may vary depending upon the particular form and rate of radiation involved, the nature and characteristics of the particular preparation of one or more digestive enzymes being irradiated and/or the particular biological contaminants or pathogens being inactivated. Suitable irradiation times can be determined empirically by one skilled in the art.

According to the methods of the present invention, the preparation of one or more digestive enzymes to be sterilized is irradiated with radiation up to a total dose effective for the inactivation of one or more active biological contaminants or pathogens in the material, while not producing an unacceptable level of damage to that material. Suitable total doses of radiation may vary depending upon certain features of the methods of the present invention being employed, such as the nature and characteristics of the particular preparation being irradiated, the particular form of radiation involved and/or the particular active biological contaminant or pathogen being inactivated. Suitable total doses of radiation can be determined empirically by one skilled in the art. Preferably, the total dose of radiation is at least 25 kGy, more preferably at

least 45 kGy, even more preferably at least 75 kGy, and still more preferably at least 100 kGy or greater, such as 150 kGy or 200 kGy or greater.

The particular geometry of the preparation of one or more digestive enzymes being irradiated, such as the thickness and distance from the source of radiation, may be determined empirically by one skilled in the art.

According to certain methods of the present invention, an effective amount of at least one sensitizing compound may optionally be added to the preparation of one or more digestive enzymes prior to irradiation, for example to enhance the effect of the irradiation on the biological contaminant(s) or pathogen(s) therein, while employing the methods described herein to minimize the deleterious effects of irradiation upon the preparation of one or more digestive enzymes. Suitable sensitizers are known to those skilled in the art, and include, for example, psoralens and their derivatives and analogs and inactines and their derivatives and analogs.

According to the methods of the present invention, the irradiation of the preparation of one or more digestive enzymes may occur at any temperature that is not deleterious to the preparation of one or more digestive enzymes being sterilized. According to one preferred embodiment, the preparation of one or more digestive enzymes is irradiated at ambient temperature. According to an alternate preferred embodiment, the preparation of one or more digestive enzymes is irradiated at reduced temperature, *i.e.* a temperature below ambient temperature, such as 0°C, -20°C, -40°C, -60°C, -78°C or -196°C. According to this embodiment of the present invention, the preparation of one or more digestive enzymes is preferably irradiated at or below the freezing or eutectic point of the preparation of one or more digestive enzymes. According to another alternate preferred embodiment, the preparation of one or more digestive enzymes is irradiated at elevated temperature, *i.e.* a temperature above ambient temperature, such as 37°C, 60°C, 72°C or 80°C. While not wishing to be bound by any theory, the use of elevated temperature may enhance the effect of irradiation on the biological contaminant(s) or pathogen(s) and therefore allow the use of a lower total dose of radiation.

Most preferably, the irradiation of the preparation of one or more digestive enzymes occurs at a temperature that protects the preparation from radiation. Suitable temperatures can be determined empirically by one skilled in the art.

In certain embodiments of the present invention, the temperature at which irradiation is performed may be found to lie within a range, rather than at a specific point. Such a range for the preferred temperature for the irradiation of a particular preparation of one or more digestive enzymes may be determined empirically by one skilled in the art.

According to the methods of the present invention, the irradiation of the preparation of one or more digestive enzymes may occur at any pressure which is not deleterious to the biological material being sterilized. According to one preferred embodiment, the preparation opf one or more digestive enzymes is irradiated at elevated pressure. More preferably, the preparation of one or more digestive enzymes is irradiated at elevated pressure due to the application of sound waves, the use of a volatile, compression or other means known to those skilled in the art. While not wishing to be bound by any theory, the use of elevated pressure may enhance the effect of irradiation on the biological contaminant(s) or pathogen(s) and/or enhance the protection afforded by one or more stabilizers, and therefore allow the use of a lower total dose of radiation. Suitable pressures can be determined empirically by one skilled in the art.

Generally, according to the methods of the present invention, the pH of the preparation of one or more digestive enzymes undergoing sterilization is about 7. In some embodiments of the present invention, however, the preparation of one or more digestive enzymes may have a pH of less than 7, preferably less than or equal to 6, more preferably less than or equal to 5, even more preferably less than or equal to 4, and most preferably less than or equal to 3. In alternative embodiments of the present invention, the preparation of one or more digestive enzymes may have a pH of greater than 7, preferably greater than or equal to 8, more preferably greater than or equal to 9, even more preferably greater than or equal to 10, and most preferably greater than or equal to 11. According to certain embodiments of the present invention, the pH of the preparation undergoing sterilization is at or near the isoelectric point of the enzyme(s) contained in the preparation. According to other embodiments of the present invention, the pH of the

preparation undergoing sterilization is at or near the pH at which at least one enzyme in the preparation has maximal affinity for its substrate(s). Suitable pH levels can be determined empirically by one skilled in the art.

Similarly, according to the methods of the present invention, the irradiation of the preparation of one or more digestive enzymes may occur under any atmosphere that is not deleterious to the preparation of one or more digestive enzymes being treated. According to one preferred embodiment, the preparation of one or more digestive enzymes is held in a low oxygen atmosphere or an inert atmosphere. When an inert atmosphere is employed, the atmosphere is preferably composed of a noble gas, such as helium or argon, more preferably a higher molecular weight noble gas, and most preferably argon. According to another preferred embodiment, the preparation of one or more digestive enzymes is held under vacuum while being irradiated. According to a particularly preferred embodiment of the present invention, a preparation of one or more digestive enzymes (lyophilized, liquid or frozen) is stored under vacuum or an inert atmosphere (preferably a noble gas, such as helium or argon, more preferably a higher molecular weight noble gas, and most preferably argon) prior to irradiation. According to an alternative preferred embodiment of the present invention, a liquid preparation of one or more digestive enzymes is held under low pressure, to decrease the amount of gas, particularly oxygen, dissolved in the liquid, prior to irradiation, either with or without a prior step of solvent reduction, such as lyophilization. Such degassing may be performed using any of the methods known to one skilled in the art.

In another preferred embodiment, where the preparation of one or more digestive enzymes contains oxygen or other gases dissolved within or associated with it, the amount of these gases within or associated with the preparation may be reduced by any of the methods and techniques known and available to those skilled in the art, such as the controlled reduction of pressure within a container (rigid or flexible) holding the preparation to be treated or by placing the preparation in a container of approximately equal volume.

It will be appreciated that the combination of one or more of the features described herein may be employed to further minimize undesirable effects upon the preparation of one or more

digestive enzymes caused by irradiation, while maintaining adequate effectiveness of the irradiation process on the biological contaminant(s) or pathogen(s). For example, in addition to the use of a stabilizer, a particular preparation of one or more digestive enzymes may also be lyophilized, held at reduced temperature and kept under vacuum prior to irradiation to further minimize undesirable effects.

The sensitivity of a particular biological contaminant or pathogen to radiation is commonly calculated by determining the dose necessary to inactivate or kill all but 37% of the agent in a sample, which is known as the D_{37} value. The desirable components of a preparation of one or more digestive enzymes may also be considered to have a D_{37} value equal to the dose of radiation required to eliminate all but 37% of their desirable biological and physiological characteristics.

In accordance with certain preferred methods of the present invention, the sterilization of a preparation of one or more digestive enzymes is conducted under conditions that result in a decrease in the D_{37} value of the biological contaminant or pathogen without a concomitant decrease in the D_{37} value of the preparation of one or more digestive enzymes. In accordance with other preferred methods of the present invention, the sterilization of a preparation of one or more digestive enzymes is conducted under conditions that result in an increase in the D_{37} value of the preparation of one or more digestive enzymes. In accordance with the most preferred methods of the present invention, the sterilization of a preparation of one or more digestive enzymes is conducted under conditions that result in a decrease in the D_{37} value of the biological contaminant or pathogen and a concomitant increase in the D_{37} value of the preparation of one or more digestive enzymes.

Examples

The following examples are illustrative, but not limiting, of the present invention. Other suitable modifications and adaptations are of the variety normally encountered by those skilled in the art and are fully within the spirit and scope of the present invention. Unless otherwise noted, all irradiation was accomplished using a ^{60}Co source.

Example 1

In this experiment, lyophilized trypsin was irradiated (45 kGy at 1.9 kGy/hr) alone or in the presence of a stabilizer (sodium ascorbate 100 mM) at varying levels of residual solvent content.

Method

1 ml aliquots of trypsin alone or with 100 mM sodium ascorbate (10 mg/ml) were placed in 3 ml vials. Samples were prepared in triplicate and subjected to lyophilization, either a primary drying cycle (22 hours, sample temp 0-10°C, shelf temp 35°C, 10 mT) or a combination of a primary drying cycle and a secondary drying cycle (60 hours, sample temp 40°C, shelf temp 40°C, 10 mT).

All samples were resuspended in 1 ml water, and then diluted 1:10 for assay. Assay conditions: 50 units/ml trypsin per well + BAPNA substrate starting at 3000 µg/ml was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

In the absence of stabilizer, lyophilized trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 74% of control activity at the higher residual solvent content level, *i.e.* about 2.4% water, and recovery of 85% of control activity at the lower residual solvent content level, *i.e.*, about 1.8% water.

In the presence of stabilizer, trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 97% of control activity at higher residual solvent content levels, *i.e.* about 3.7% water, and recovery of 86% of control activity at lower residual solvent content levels, *i.e.* about 0.7% water.

The results of this experiment are shown graphically in Figures 1A-1B.

Example 2

In this experiment, trypsin was irradiated (45 kGy at 1.6 kGy/hr. and 4°C) in the presence of a stabilizer (sodium ascorbate 200 mM) as either a liquid or lyophilized preparation at varying pH levels.

Method

1 ml of 1 mg/ml (about 3000 IU/ml) trypsin aliquots in the presence of 35 mM phosphate buffer and 200 mM sodium ascorbate were made at varying pH levels between 5 and 8.5, inclusive. 400 µl of each solution was placed in 3 ml vials and then lyophilized and gamma-irradiated. The remaining portion of each solution was gamma-irradiated as a liquid. Lyophilized and liquid samples were assayed at the same time, under the following conditions: Assay conditions: 5 U/well trypsin (50 U/ml) + BATNA substrate (1 mg/ml) was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

Liquid trypsin samples exposed to 45 kGy total dose gamma-irradiation showed recovery of between about 70 and 75% of control activity across the pH range tested. Lyophilized trypsin samples showed recovery of between about 86 and 97% of control activity across the same pH ranges. More specifically, the following results were observed:

Sample #	pH	lyophilized (% of control activity)	liquid (% of control activity)
1	5	91.11	69.87
2	5.5	94.38	74.86
3	6	85.54	75.77
4	6.47	96.26	71.79
5	7	90.40	75.59
6	7.5	96.79	75.63
7	7.8	90.62	74.55
8	8.5	89.59	71.08

The results of this experiment are shown graphically in Figure 2.

Example 3

In this experiment, lyophilized trypsin was irradiated (42.7-44.8 kGy at 2.65 kGy/hr at 4°C) alone or in the presence of a stabilizer (sodium ascorbate 200 mM).

Method

1 ml aliquots of trypsin alone or with 200 mM sodium ascorbate (1 mg/ml) were placed in 3 ml vials and frozen overnight at -70°C. Samples were prepared in quadruplicate and subjected to lyophilization, utilizing primary and secondary drying cycles (20 hours total).

All samples were resuspended in 1 ml water, and then diluted 1:10 for assay. Assay conditions: 50 units/ml trypsin per well + BATNA substrate starting at 3000 µg/ml was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this

value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

In the absence of stabilizer, lyophilized trypsin exposed to gamma-irradiation showed recovery of 63% of control activity. In the presence of stabilizer, lyophilized trypsin exposed to gamma-irradiation showed recovery of 88% of control activity. The results of this experiment are shown graphically in Figures 3A-3B.

Example 4

In this experiment, trypsin that had been lyophilized (0.7% moisture) was irradiated (45 kGy at 1.867 kGy/hr at 3.2°C) alone or in the presence of a stabilizer (sodium ascorbate 100 mM) at varying levels of residual solvent content.

Method

1 ml aliquots of trypsin alone or with 100 mM sodium ascorbate (10 mg/ml) were placed in 3 ml vials and frozen overnight at -70°C. Samples were prepared in quadruplicate and subjected to lyophilization (69.5 hours total run time; shelf temperature 35°C).

All samples were resuspended in 1 ml water, and then diluted 1:10 for assay. Assay conditions: 50 units/ml trypsin per well + BAPNA substrate starting at 3000 μg/ml was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

In the absence of stabilizer, trypsin (3.9% water) exposed to 45 kGy total dose gamma-irradiation showed recovery of 77% of control activity. In the presence of stabilizer, trypsin (0.7% water) exposed to 45 kGy total dose gamma-irradiation showed recovery of 86% of control activity. The results of this experiment are shown graphically in Figures 4A-4B.

Example 5

In this experiment, lyophilized trypsin was irradiated (45 kGy at 1.9 kGy/hr) alone or in the presence of a stabilizer (sodium ascorbate 100 mM) at varying levels of residual solvent content.

Method

1 ml aliquots of trypsin alone or with 100 mM sodium ascorbate (10 mg/ml) were placed in 3 ml vials. Samples were prepared in triplicate and subjected to lyophilization, either a primary drying cycle (25 hours, sample temp 0-10°C, shelf temp 35°C, 10 mT) or a combination of a primary drying cycle and a secondary drying cycle (65 hours, sample temp 40°C, shelf temp 40°C, 10 mT).

All samples were resuspended in 1 ml water, and then diluted 1:10 for assay. Assay conditions: 50 units/ml trypsin per well + BAPNA substrate starting at 3000 μg/ml was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

In the absence of stabilizer, trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 74% of control activity at the higher residual solvent content level, *i.e.* about 5.8% water, and recovery of 77% of control activity at the lower residual solvent content level, *i.e.*, about 5.4% water.

In the presence of stabilizer, trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 97% of control activity at higher residual solvent content levels, *i.e.* about 2.8% water, and recovery of 90% of control activity at lower residual solvent content levels, *i.e.* about 1.1% water.

The results of this experiment are shown graphically in Figures 5A-5B.

Example 6

In this experiment, trypsin suspended in polypropylene glycol 400 was subjected to gamma irradiation at varying levels of residual solvent (water) content.

Method

Trypsin was suspended in polypropylene glycol 400 at a concentration of about 20,000 U/ml and divided into multiple samples. A fixed amount of water (0%, 1%, 2.4%, 4.8%, 7%, 9%, 10%, 20%, 33%) was added to each sample; a 100% water sample was also prepared which contained no PPG 400.

Samples were irradiated to a total dose of 45 kGy at a rate of 1.9 kGy/hr and a temperature of 4°C. Following irradiation, each sample was centrifuged to pellet the undissolved trypsin. The PPG/water soluble fraction was removed and the pellets resuspended in water alone.

Assay conditions: 5 U/well trypsin (50 U/ml) + BAPNA substrate (0.5 mg/ml) was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

The irradiated samples containing a mixture of polypropylene glycol (PPG 400) and water (up to 33% water) retained about 80% of the activity of an unirradiated trypsin control and activity equal to that of a dry (lyophilized) trypsin control irradiated under identical conditions. No activity was detected in the 100% water sample irradiated to 45 kGy. The results of this experiment are shown graphically in Figure 6.

Example 7

In this experiment, an aqueous solution of trypsin was subjected to gamma irradiation at varying concentrations of a stabilizer (sodium ascorbate, alone or in combination with 1.5mM uric acid).

Method

Trypsin samples (5 Units/sample) were prepared with varying concentrations of sodium ascorbate, alone or in combination with 1.5mM uric acid. Samples were irradiated to a total dose of 45 kGy at a rate of 1.9 kGy/hr and a temperature of 4°C.

Assay conditions: 5 U/well trypsin (50 U/ml) + 50 µl BAPNA substrate (1 mg/ml). The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15

minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

The irradiated samples containing at least 20mM ascorbate retained varying levels of trypsin activity compared to an unirradiated control. Samples containing 125mM or more ascorbate retained about 75% of the trypsin activity of an unirradiated control. Similar results were observed with samples containing ascorbate in combination with uric acid. The results of this experiment are shown graphically in Figure 7.

Example 8

In this experiment, the protective effect of ascorbate (200 mM) and a combination of ascorbate (200 mM) and Gly-Gly (200 mM) on two different frozen enzyme preparations (a glycosidase and a sulfatase) was evaluated.

Method

In glass vials, 300 µl total volume containing 300 µg of enzyme (1 mg/ml) were prepared with either no stabilizer or the stabilizer of interest. Samples were irradiated with gamma radiation (45 kGy total dose, dose rate and temperature of 1.616 kGy/hr and -21.5°C or 5.35 kGy/hr and -21.9°C) and then assayed for structural integrity.

Structural integrity was determined by SDS-PAGE. Three 12.5% gels were prepared according to the following recipe: 4.2 ml acrylamide; 2.5 ml 4X-Tris (pH 8.8); 3.3 ml water; 100 µl 10% APS solution; and 10µl TEMED. This solution was then placed in an electrophoresis unit with 1X Running Buffer (15.1 g Tris base; 72.0 g glycine; 5.0 g SDS in 1 l water, diluted 5-fold). Irradiated and control samples (1 mg/ml) were diluted with Sample Buffer (+/- beta-ME) in Eppindorf tubes and then centrifuged for several minutes. 20µl of each diluted sample (~10 µg) were assayed.

Results

Liquid enzyme samples irradiated to 45 kGy in the absence of a stabilizer showed significant loss of material and evidence of both aggregation and fragmentation. Much greater recovery of material was obtained from the irradiated samples containing ascorbate or a combination of ascorbate and Gly-Gly. The results of this experiment are shown in Figures 8A-8B.

Example 9

In this experiment, the protective effect of ascorbate (200 mM) and a combination of ascorbate (200 mM) and Gly-Gly (200 mM) on a frozen glycosidase preparation was evaluated.

Method

Samples were prepared in 2 ml glass vials, each containing 52.6 µl of a glycosidase solution (5.7 mg/ml), and either no stabilizer or a stabilizer of interest, and sufficient water to make a total sample volume of 300 µl. Samples were irradiated with gamma radiation (45 kGy total dose, dose rate and temperature of either 1.616 kGy/hr and -21.5°C or 5.35 kGy/hr and -21.9°C) and then assayed for structural integrity.

Structural integrity was determined by reverse phase chromatography. 10 µl of sample were diluted with 90 µl solvent A and then injected onto an Aquapore RP-300 (c-8) column (2.1 x 30 mm) mounted in an Applied Biosystems 130A Separation System Microbore HPLC. Solvent A: 0.1% trifluoroacetic acid; solvent B: 70% acetonitrile, 30% water, 0.085% trifluoroacetic acid.

Results

Enzyme samples irradiated to 45 kGy in the absence of a stabilizer showed broadened and reduced peaks. Much greater recovery of material, as evidenced by significantly less

reduction in peak size compared to control (Figure 9), was obtained from the irradiated samples containing ascorbate or a combination of ascorbate and Gly-Gly.

Example 10

In this experiment, lyophilized trypsin was irradiated (45 kGy total dose at 1.9 kGy/hr. at 4°C) in the presence of Tris buffer (pH 7.6) or phosphate buffer (pH 7.5).

Method

Aliquots of a 1000 IU/ml trypsin solution were placed in 3 ml vials and then lyophilized and gamma-irradiated. The remaining portion of each solution was gamma-irradiated as a liquid. Samples were assayed under the following conditions: Assay conditions: 5 U/well trypsin (50 U/ml) + BATNA substrate (1 mg/ml) was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

Lyophilized trypsin samples exposed to 45 kGy total dose gamma-irradiation showed recovery of essentially all trypsin activity in the presence of Tris buffer and sodium ascorbate and recovery of 88% of trypsin activity in the presence of phosphate buffer and sodium ascorbate.

Example 11

In this experiment, lyophilized enzyme preparations (a glycosidase and a sulfatase) were irradiated in the absence or presence of a stabilizer (100 mM sodium ascorbate).

Method

Glass vials containing 1 mg of enzyme were prepared with either no stabilizer or 100 mM sodium ascorbate (50µl of 2M solution) and sufficient water to make 1 ml of sample. Samples were lyophilized following moisture levels: glycosidase with stabilizer, 3.4%; glycosidase without stabilizer, 3.2%; sulfate with stabilizer, 1.8%; and sulfate without stabilizer, 0.7%. Lyophilized samples were irradiated with gamma radiation (45 kGy total dose at 1.8 kGy/hr and 4°C) and then assayed for structural integrity.

Structural integrity was determined by SDS-PAGE. In an electrophoresis unit, 6 µg/lane of each sample was run at 120V on a 7.5%-15% acrylamide gradient gel with a 4.5% acrylamide stacker under non-reducing conditions.

Results

Lyophilized glycosidase samples irradiated to 45 kGy in the absence of a stabilizer showed significant recovery of intact enzyme with only some fragmentation. Fragmentation was reduced by the addition of a stabilizer.

Similarly, lyophilized sulfatase samples irradiated to 45 kGy in the absence of a stabilizer showed good recovery of intact enzyme, but with slightly more fragmentation. Fragmentation was again reduced by the addition of a stabilizer.

The results of this experiment are shown in Figure 10.

Example 12

In this experiment, lyophilized glycosidase preparations irradiated in the absence or presence of a stabilizer (200 mM sodium ascorbate or a combination of 200 mM ascorbate and 200 mM glycylglycine).

Methods

Samples were prepared in glass vials, each containing 300 µg of a lyophilized glycosidase and either no stabilizer or a stabilizer of interest. Samples were irradiated with gamma radiation to varying total doses (10 kGy, 30 kGy and 50 kGy total dose, at a rate of 0.6 kGy/hr. and a temperature of -60°C) and then assayed for structural integrity using SDS-PAGE.

Samples were reconstituted with water to a concentration of 1 mg/ml, diluted 1:1 with 2x sample buffer (15.0 ml 4x Upper Tris-SDS buffer (pH 6.8); 1.2 g sodium dodecyl sulfate; 6 ml glycerol; sufficient water to make up 30 ml; either with or without 0.46g dithiothreitol), and then heated at 80°C for 10 minutes. 10 µl of each sample (containing 5 µg of enzyme) were loaded into each lane of a 10% polyacrylamide gel and run on an electrophoresis unit at 125V for about 1.5 hours.

Results

About 80% of the enzyme was recovered following irradiation of the samples containing no stabilizer, with some degradation as shown in Figures 11A-11C. Less degradation was observed in the samples containing ascorbate alone as the stabilizer, and even less degradation in the samples containing a combination of ascorbate and glycylglycine as the stabilizer.

Having now fully described this invention, it will be understood to those of ordinary skill in the art that the methods of the present invention can be carried out with a wide and equivalent range of conditions, formulations, and other parameters without departing from the scope of the invention or any embodiments thereof.

All patents and publications cited herein are hereby fully incorporated by reference in their entirety. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that such publication is prior art or that the present invention is not entitled to antedate such publication by virtue of prior invention.